REMARKS

The February 19, 2003 Official Action and references cited therein have been carefully reviewed. In light of the amendments presented herewith and the following remarks, favorable reconsideration and allowance of the application are respectfully requested.

At the outset, Applicants hereby request the references cited in the parent application, U.S. Application 09/046,373, be considered and printed on the face of any patent which issues from the above-identified application.

At page 2 of the Official Action, the Examiner acknowledges Applicants' election without traverse of claims 6, 8, 12, and 13 for further prosecution.

At page 2, the Examiner indicates that the abbreviations "Ab" and "Ag" are used in the instant specification without definition. Applicants submit that the skilled person is well-aware of the meaning of these abbreviations. However, this omission has been corrected by the amendment to the specification presented hereinabove. The Examiner also indicates that "ΔG_{TS}" was referred to in the description of Figure 1 but not shown on Figure 1 and that "ΔG_{rs}" and "ΔG_f" are shown on Figure 1 but not defined in the description. It is submitted that the "ΔG_{rs}" shown on Figure 1, which the Examiner refers to, is actually "ΔG_{TS}", which has been defined in the description. The "ΔG_f" shown on Figure 1, which the Examiner refers to, is actually "ΔG_p". To eliminate this confusion, Applicants submit herein a replacement of Figure 1 which clearly indicates "ΔG_{TS}" and "ΔG_p". Additionally, the description of Figure 1 has been amended by the foregoing amendment to include a definition of "ΔG_p". The skilled person of transition state chemistry is well aware of the meaning of this term which appeared in the specification as originally filed. Accordingly,

applicants submit that the amendment to the description of Figure 1 does not introduce new matter into the specification. In light of the foregoing amendments, the objections to the specification and figures are now moot.

Also at page 2, the Examiner rejects claims 6, 8, 12, and 13 under 35 U.S.C. §112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

At page 3, the Examiner further rejects claims 6, 8, 12, and 13 under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention and in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

At page 5, the Examiner rejects claims 6 and 8 under 35 U.S.C. §103(a) as allegedly being unpatentable over Lerner, R.A., et al., (1991) *Science* 252:659-667.

Finally, at page 6, claim 12 is rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over either Blackburn, G.M., et al. (1996) *Pure & Appl. Chem.* 68(11):2009-2016 or Landry (U.S. Patent 5,948,658).

The foregoing constitutes the entirety of the objections and rejections raised in the February 19, 2003 Official Action. In light of the present amendments and the following remarks, each of the above-noted rejections under 35 U.S.C. §§112, first and second paragraphs, and 103(a) is respectfully traversed.

The metes and bounds of claims 6, 8, 12, and 13 as amended are clear

The Examiner has rejected claims 6, 8, 12, and 13 under U.S.C. §112, second paragraph. Specifically, the Examiner asserts that the phrase "covalently reactive antigen analog" in claim 6 is not defined in the claim. Additionally, the Examiner has construed the phrase to mean "something that is bound covalently to an antigen with a reactive analog". It is submitted that the Examiner's position is erroneous and the term "covalently reactive antigen analog (CRAA)" has been defined in the present specification. See page 16, lines 14-20, where it is disclosed that the CRAAs of the invention are novel antigen analogs which "contain an electrophilic center flanked by peptide residues derived from proteins associated with a particular peptide antigen to be targeted for cleavage and the intended use of the CRAA". The specification further provides the structure of several exemplary CRAAs, see figures 10, 15 and 16. As set forth in the present application, the instantly claimed CRAA of the invention are superior to conventionally used TSA because they contain essential elements which act in concert to a) bind chemically reactive serine residues encoded by the germline genes for certain serine protease types of catalytic antibodies (as well as residues such as Thr and Cys that might acquire their chemical reactivity via somatic sequence diversification of the germline genes); b) utilize ion pairing and noncovalent forces to bind structures such as positively charged Asp/Glu residues that are responsible for the basic residue cleavage specificity of the germline encoded catalytic sites; and c) bind antibody combining sites at multiple amino acids via ion pairing and non-covalent forces. See page 6, lines 5-18. Thus, the term "covalently reactive antigen analog" (CRAA) is not only well defined, but structural

examples of such analogs are exemplified in the specification. Accordingly, it cannot be reasonably maintained that the subject matter of claim 6 is indefinite.

The Examiner further states that the recitation of "CRAA" in claim 8 lacks proper antecedent basis. This has been corrected by the amendment to claim 6, from which claim 8 depends, wherein "CRAA" is defined as the abbreviation of "covalently reactive antigen analog". Also, in response to the Examiner's assertion that the abbreviation of "CRAA" is not defined in claim 13, Applicants present hereinwith an amendment to claim 13 wherein the omission is corrected.

Additionally, by the foregoing claim amendment, claim 12 has been amended to correct the misspelling of words "administering" and "catalytic antibodies".

Therefore, it is submitted that the metes and bounds of claims 6, 8, 12, and 13, as amended are clear. Accordingly, the rejections of these claims under U.S.C. §112, second paragraph, should be withdrawn.

Claims 6, 8, 12, and 13 Have Met the Requirement under 35 U.S.C. §112, First Paragraph

At page 3, the Examiner has rejected claims 6, 8, 12 and 13 as allegedly not complying with the written description and enablement requirements of 35 U.S.C. §112, first paragraph. It is submitted that this rejection is improper and should be withdrawn.

Claims 6, 8, 12, and 13 are directed to methods for producing catalytic antibodies using the CRAAs of the present invention, methods for passively immunizing patients using the catalytic antibodies produced therefrom, and methods for actively immunizing patients using vaccines comprising the CRAAs of the present invention. An enabled description of the claimed

methods has been fully provided in the specification as originally filed. For example, at page 29, lines 1-10, a method of stimulating catalytic antibody synthesis by immunizing mice with an electrophilic CRAA of an EGFR peptide is disclosed. At the paragraphs that follow (at pages 29-40), a detailed description of materials and methods used in practicing the methods are also provided, e.g., preparation of EGFR-CRAA peptide; immunization of mice; EGFR-CRAA ELISA; catalytic selection reagents; screening for catalytic activity; and assessment of catalytic properties. Also, at pages 88-102 of the specification, protocols used for stimulating the production of anti-gp120 catalytic antibodies by immunizing mice with gp120-CRAA of the present invention are disclosed. Further, in Examples IV and V (pages 121-123) of the specification, methods of passive immunization with the catalytic antibodies of the present invention and methods of active immunization with the CRAAs of the present invention are described. Thus, the subject matter of claims 6, 8, 12, and 13 is fully described by the specification as it was originally filed.

With regards to the Examiner's position that the claimed inventions are not enabled by the specification, it is respectfully submitted that the Examiner has failed to establish a *prima facie* case of unenablement. Specifically, in *Fiers v. Sugano*, 984 F.2d 1164, 25 USPQ 2d 1601,1607 (Fed. Cir. 1993) the Federal Circuit has stated:

> "[A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of §112 *unless* there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. …"
> "[A]ny party making the assertion that a U.S. patent

specification or claims fails, for one reason or another, to comply with §112 bears the burden of persuasion in showing said lack of compliance."

Moreover, in *Staehelin v. Secher*, the Board provides:

"It has been consistently held that the first paragraph of 35 USC 112 required nothing more than objective enablement. … However such a teaching is set forth, whether by the use of illustrative examples or by broad descriptive terminology, is of no importance since a specification which teaches how to make and use the invention in terms which correspond in scope to the claims *must* be taken as complying with the first paragraph of 35 USC 112 *unless* there is reason to doubt the objective truth of the statements relied upon therein for enabling support."

In the instant case, lacking a specific showing, the mere assertion by the Examiner that the production of catalytic antibodies is unpredictable does not render the claimed invention unenabled.

Additionally, submitted hereinwith is a recent publication by a co-inventor of the present invention, Sudhir P. et al., "Specific HIV gp120-cleaving Antibodies Induced by Covalently Reactive Analog of gp120", *The Journal of Biological Chemistry* 2003;278(22):20429-20435. In this publication, 7 monoclonal antibodies were raised by immunization with a covalently reactive antigen analog of gp120 having a similar structure with the CRAA-gp120 of the present invention. Catalytic cleavage of biotinylated gp120 by 3 of the 7 monoclonal antibodies is observed. Sudhir P. et al., therefore, provide further evidence that the claimed invention is enabled.

The examiner has concluded that the subject matter of claim 13 lacks enablement. There are many factors to be considered when determining whether there is sufficient evidence to support a determination that a disclosure does not satisfy the

enablement requirement and whether any necessary factors experimentation is "undue". These factors include

(a) The breadth of the claims;
(b) The nature of the invention;
(c) The state of the art;
(d) The level of one of ordinary skill;
(e) The amount of direction provided by the inventors;
(f) The existence of working examples;
(g) The quantity of experimentation needed to make or use the invention based on the content of the disclosure.

A conclusion of lack of enablement means that, based on the evidence regarding each of the above factors, the specification, at the time the application was filed, would not have taught one skilled in the art how to make and/or use the full scope of the claimed invention with out undue experimentation (*In re Wright*, 999 F.2d 1557,1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993).

Applicants submit that in light of the evidence provided above, regarding the generation of catalytic antibodies using covalently reactive analogs for gp120, the disclosure in specification relating to immunization with CRAA, and the general knowledge of those skilled in the art of immunization, it is clear that the skilled person could practice the subject matter encompassed by claim 13 without undue experimentation. Based on the above, it is submitted that the subject matter of claims 6, 8, 12, and 13 is fully described and enabled by the specification as it was originally filed. Accordingly, the foregoing rejections under §112, first paragraph, are improper and should be withdrawn.

Claims 6 and 8 are not obvious over Lerner et al.

Applicants strenuously disagree with the Examiner's assertion, at page 5 of the Official Action, that claims 6 and 8 are obvious over Lerner et al.

First, the Examiner, here again, asserts "the meaning of 'covalently reactive antigen analog' is not defined" and that "it is taken to mean that something is bound covalently to an antigen with a reactive analog". As discussed above, the meaning of "covalently reactive antigen analog" (CRAA) has been clearly defined in the specification. The CRAAs of the present invention are antigen analogs comprising an electrophilic center flanked by peptide residues derived from proteins associated with a particular peptide antigen to be targeted for cleavage. CRAAs can be used to stimulate the production of catalytic antibodies against peptide antigens.

Second, to establish a *prima facie* case of obviousness, three basic criteria must be met: (1) there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings; (2) there must be a reasonable expectation of success; and (3) the prior art reference (or references when combined) must teach or suggest all the claimed limitations (MPEP §2143).

In the instance case, claims 6 and 8 are directed to methods for producing catalytic antibodies by administering to test subjects covalently reactive antigen analogs (CRAAs). These methods, especially the use of CRAAs, are neither taught or suggested by Lerner et al.

Particularly, the Examiner's assertion that "[t]he instant reference teaches the use of several antigen having analogs to the substrate to produce catalytic antibodies" and therefore

"[i]t would have been obvious to one of ordinary skill in the art to administer the antigens to a test subject with the anticipation that catalytic antibodies would be produced" is erroneous.

Lerner et al., in fact, teach the discovery and characterization of a unique type of antibodies, catalytic antibodies. While the reference also provides "[a] number of general strategies … for generating catalytic antibodies, including (i) the use of antibodies to stabilize negatively and positively charged transition states, (ii) the use of antibodies as entropic traps, and (iii) the generation of antibodies with catalytic groups and cofactors in their combining sites." (Page 660, left column) Lerner et al., nowhere, teach or suggest "the use of antigen analogs having the features of the instantly claimed CRAA. Thus the reference fails to provide the skilled person with the motivation to develop the methods encompassed by claims 6 or 8 or the expectation of success of such methods. Accordingly, claims 6 and 8 are not unpatentable over Lerner et al.

Claim 12 as amended is not obvious over either Blackburn et al. or Landry

Claim 12 has been amended to recite methods for passively immunizing a patient with catalytic antibodies that are produced using the CRAAs of the present invention.

However, Landry teaches catalytic antibodies capable of degrading cocaine and the use thereof for treating cocaine overdose and addiction in subjects. Blackburn et al. also teach the use of specific catalytic antibodies, such as those disclosed in Landry, in the clearance of toxic substances from the blood stream. Blackburn also teach the use of catalytic antibodies in converting prodrugs into active agents. It is

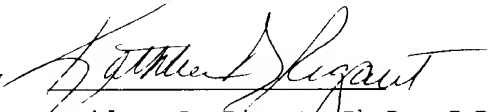
noted, however, that none of the cited references, teaches or suggests the use of catalytic antibodies produced using CRAAs of the present invention. Thus, neither Landry or Blackburn et al. teach or suggest the method encompassed by claim 12. It is a well-settled premise in patent law that "silence in a reference is not a proper substitute for adequate disclosure of facts from which a conclusion of obviousness may justifiably follow". In re Burt, 148 U.S.P.Q. 548 (CCPA 1966).

Applicants submit that the obviousness rejection of claim 12 over Landry or Blackburn et al. is therefore improper and should be withdrawn.

CONCLUSION

In view of the amendments presented herewith and the foregoing remarks, it is respectfully urged that the rejections set forth in the February 19, 2003 Official Action be withdrawn and that this application be passed to issue. In the event the Examiner is not persuaded as to the allowability of any claim, and it appears that any outstanding issues may be resolved through a telephone interview, the Examiner is requested to telephone the undersigned attorney at the phone number given below.

Respectfully submitted,
DANN DORFMAN HERRELL & SKILLMAN, P.C.

By 
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THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 278, No. 22, Issue of May 30, pp. 20429–20435, 2003
Printed in U.S.A.

Specific HIV gp120-cleaving Antibodies Induced by Covalently Reactive Analog of gp120*

Received for publication, January 27, 2003, and in revised form, March 26, 2003
Published, JBC Papers in Press, March 28, 2003, DOI 10.1074/jbc.M300870200

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We report the results of efforts to strengthen and direct the natural nucleophilic activity of antibodies (Abs) for the purpose of specific cleavage of the human immunodeficiency virus-1 coat protein gp120. Phosphonate diester groups previously reported to form a covalent bond with the active site nucleophile of serine proteases (Paul, S., Tramontano, A., Gololobov, G., Zhou, Y. X., Taguchi, H., Karle, S., Nishiyama, Y., Planque, S., and George, S. (2001) *J. Biol. Chem.* 276, 28314–28320) were placed on Lys side chains of gp120. Seven monoclonal Abs raised by immunization with the covalently reactive analog of gp120 displayed irreversible binding to this compound (binding resistant to dissociation with the denaturant SDS). Catalytic cleavage of biotinylated gp120 by three monoclonal antibodies was observed. No cleavage of albumin and the extracellular domain of the epidermal growth factor receptor was detected. Cleavage of model peptide substrates occurred on the C-terminal side of basic amino acids, and K_m for this reaction was ~200-fold greater than that for gp120 cleavage, indicating Ab specialization for the gp120 substrate. A hapten phosphonate diester devoid of gp120 inhibited the catalytic activity with exceptional potency, confirming that the reaction proceeds via a serine protease mechanism. Irreversible binding of the hapten phosphonate diester by polyclonal IgG from mice immunized with gp120 covalently reactive analog was increased compared with similar preparations from animals immunized with control gp120, indicating induction of Ab nucleophilicity. These findings suggest the feasibility of raising antigen-specific proteolytic antibodies on demand by covalent immunization.

Promiscuous cleavage of small peptide substrates is a heritable function of Abs[1] encoded by germ line gene variable domains (for review, see Ref. 1). Peptide bond cleaving Abs with specificity for individual polypeptides have been identified in patients with autoimmune (1) and alloimmune disease (2). Specific monoclonal Abs and Ab light chain subunits displaying proteolytic activities can be raised by routine immunization with polypeptides (3, 4). Under ordinary circumstances, however, adaptive maturation of the catalytic activity may not be a favored event. B cell clonal selection occurs by sequence diversification of genes encoding the Ab variable domains followed by selective binding of the antigen to cell surface Abs with the greatest affinity, which drives proliferation of the B cells (5). Catalysis entails chemical transformation of the antigen and release of products from the Ab, which may cause cessation of B cell proliferation when the catalytic rate exceeds the rate of transmembrane signaling necessary to stimulate cell proliferation.

Originally developed as irreversible inhibitors of conventional serine proteases, haptenic phosphonate esters are reported to bind the nucleophilic sites of natural proteolytic Abs covalently (6, 7). The haptenic phosphonates could potentially serve as covalently reactive analogs (CRAs) for inducing the synthesis of Abs with improved nucleophilicity. To the extent that Ab nucleophilicity is rate-limiting in proteolysis, its enhancement may permit more rapid peptide bond cleavage, *i.e.* if the subsequent steps in the catalytic reaction cycle (hydrolysis of the acyl-Ab complex and product release) do not pose significant energetic hurdles (see Fig. 1). The innate character of Ab nucleophilic reactivity is the central element of this approach, and there is no requirement for *de novo* formation of chemically reactive sites over the course of variable domain sequence diversification. Most previous attempts to program the structure of catalytic sites in Abs in comparison have relied on noncovalent stabilization of the oxyanionic transition state (*i.e.* by immunization with transition state analogs; Refs. 8 and 9). An Ab with esterase activity (10) and another with aldolase activity (11) utilize covalent catalytic mechanisms, but the relationship of these activities to innate Ab nucleophilicity is unclear.

An ideal antigen-specific proteolytic Ab may be conceived to combine traditional noncovalent binding interactions in the ground state of the Ab-antigen complex with nucleophilic attack on the peptide backbone. The ground state interactions are desirable to obtain specificity for individual polypeptide antigens. No impediments for catalysis are presented by the stable ground state complexes, provided the noncovalent interactions are carried over into the transition state complex and are properly coordinated with nucleophilic attack at the reaction center. In theory, synthesis of antigen-specific proteolytic Abs could be induced by an analog that presents a mimetic of the chemical reaction center in the context of classical antigenic epitopes available for noncovalent binding interactions. If the reaction proceeds by a lock-and-key stereochemical mech-

* This work was supported by National Institutes of Health Grants AI46029 and AI31268. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: Ab, antibody; mAb, monoclonal Ab; Bt, biotin; CRA, covalently reactive antigen analog; MCA, methylcoumarinamide; VIP, vasoactive intestinal peptide; HIV, human immunodeficiency virus; exEGFR, extracellular domain of enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay.

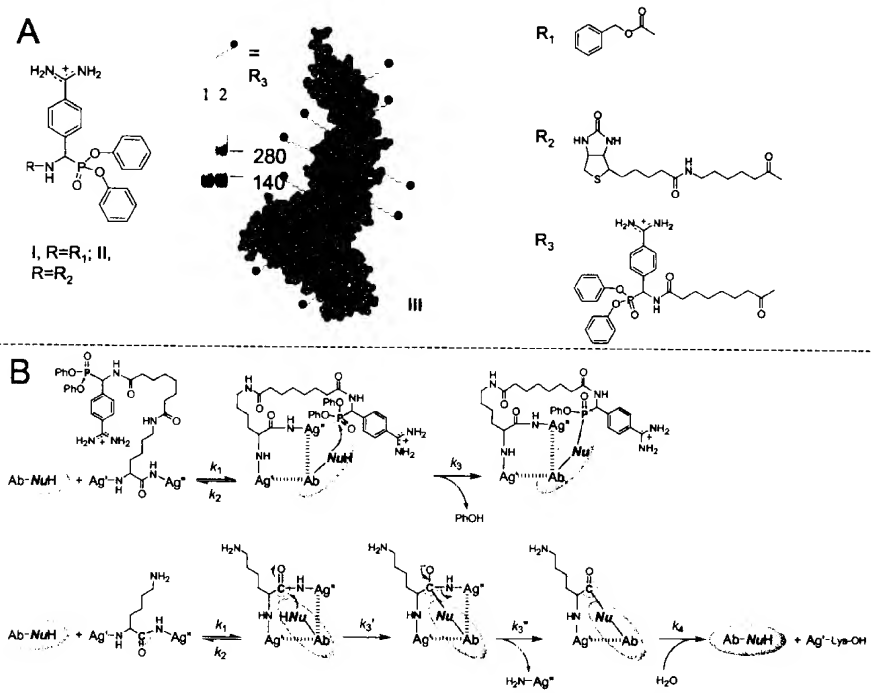

FIG. 1. **CRA structures (*A*) and their reaction with Abs (*B*).** **III** is a schematic representation of gp120 with R3 substituents at Lys residues. Left of **III** are streptavidin-peroxidase stained blots of SDS-electrophoresis gels showing biotinylated **III** containing 4 mol (*lane 1*) and 14 mol (*lane 2*) of phosphonate diester groups/mol gp120. *B*: *Nu*, nucleophile; Ag'-Lys-OH, N-terminal antigen fragment; NH_2-Ag", C-terminal antigen fragment; $k_{cat} = k3' + k3"$. A catalytic Ab forms the initial noncovalent complex by conventional epitope-paratope interactions. The active site nucleophile attacks the carbonyl carbon of the scissile bond in Ag^{2+} (substrate) to form the tetrahedral transition-state complex. The C-terminal antigen fragment is released, and the acyl-Ab complex is formed. Hydrolysis of the acyl-Ab complex results in release of the N-terminal antigen fragment and regeneration of the catalytic Ab. The reaction with phosphonate-containing Ag^{2+} recapitulates the interactions in the ground and transition state Ab-Ag^{2+} complexes (noncovalent binding at peptide epitopes and nucleophilic attack by the Ab), but unlike the acyl-Ab intermediate, the phosphonyl-Ab adduct is a stable product. A potential weakness is that immunogen **III** does not contain structural features favoring synthesis of Abs capable of rapid hydrolysis of the acyl-Ab intermediate and product release (*bottom reaction scheme*).

anism, the mimetic must be located precisely at the position of the intended scissile bond in the backbone of the polypeptide antigen. In the instance of large proteins, locating the mimetic within the protein backbone is outside the range of present-day synthetic technologies. A potential solution is to place the mimetic group at amino acid side chains using chemical linker techniques. An Ab nucleophile that recognizes the side chain mimetic could facilitate proteolysis if it enjoys sufficient conformational freedom to approach the polypeptide backbone of the substrate and form the acyl-Ab complex (see Fig. 1).

We describe here the characteristics of Abs induced by a CRA of the HIV-1 coat protein gp120 (gp120-CRA) consisting of phosphonate diester groups located in Lys side chains of the protein. Enhanced serine protease-like nucleophilic reactivity of the Abs was observed. One monoclonal Ab cleaved gp120 slowly and specifically, it displayed preference for cleavage on the C-terminal side of Lys/Arg residues, and the catalytic reaction was susceptible to CRA inhibition. These findings are the first indications that Abs with proteolytic activity specific for individual proteins can be raised on demand.

MATERIALS AND METHODS

Hapten, gp120-CRAs, and Biotinylated Proteins—Synthesis of hapten CRAs **I** and **II** (see Fig. 1) and their characterization by electrospray ionization-mass spectroscopy and elemental analyses have been described previously (12). For preparation of gp120-CRA **III**, the precursor diphenyl-*N*-[*O*-(3-sulfosuccinimidyl)suberoyl]amino-(4amidinophenyl)methanephosphonate (**IV**) was synthesized by mixing a solution of diphenyl-amino(4-amidinophenyl)methanephosphonate (79 mg, 0.13 mmol) in *N,N*-dimethylformamide (2 ml) containing *N,N*-diisopropylethylamine (0.11 ml, 0.63 mmol) and bis(sulfosuccinimidyl)suberate disodium salt (150 mg, 0.26 mmol; Pierce) for 2 h. **IV** was obtained by reversed phase high performance liquid chromatography (12) and lyophilized to give a colorless powder (yield 54%, 50 mg; *m/z* 715 (MH^+) by electrospray ionization mass spectroscopy). **IV** (1.1 mg) was reacted with electrophoretically pure gp120 (0.5 mg; Immunodiagnostic Inc., MN strain purified from baculovirus expression system) in 5 ml of 10 mM HEPES, 25 mM NaCl, 0.1 mM CHAPS, pH 7.5 buffer (2 h, 25 °C). Excess **IV** was removed by gel filtration (Micro Bio-Spin 6 disposable column, Bio-Rad), and the concentration of free amines in the initial protein and CRA-derivitized protein was measured using fluorescamine (13). The density of labeling was varied as needed from 4.0 to 32.6 mol of CRA/mol of gp120 by varying the concentration of **IV**. Preparation of gp120 labeled at Lys residues with biotin (Bt-gp120) was

by similar means using 6-biotinamidohexanoic acid *N*-hydroxysuccinimide ester (Sigma). The reaction time and reactant concentrations were controlled to yield biotin/gp120 molar ratios 0.8–1.9. Unreacted biotinylation reagent was removed using a disposable gel filtration column in 50 mM Tris-HCl, 100 mM glycine, 0.1 mM CHAPS, pH 7.8. The biotin content was determined using 2-(4′-hydroxyazobenzene)benzoic acid (14). Total protein measurements were done using the BCA method (Pierce kit). Biotinylated **III** was prepared from Bt-gp120 as described for **III**. With increasing incorporation of the hapten groups, biotinylated **III** tended to form dimers and trimers evident in SDS electrophoresis gels as bands at ~ 240 and 380 kDa (nominal mass of monomer gp120, 120 kDa). Biotinylated **III** at hapten density similar to the non-biotinylated **III** employed as immunogen (23 mol/mol of gp120) contained the monomer, dimer, and trimer species at proportions of 50, 21, and 29%, respectively. Protein-CRAs were lyophilized and stored at −20 °C until used. Bt-gp120 was stored at −70 °C in 50 mM Tris-HCl, pH 8.0, 0.1 M glycine, 0.1 mM CHAPS. Storage of **I** and **II** was at −70 °C as 10 mM solutions in *N,N*-dimethylformamide. The extracellular domain of EGFR (exEGFR) obtained from Dr. Maureen O'Connor (15) was biotinylated as described for gp120 (0.9 mol of biotin/mol of exEGFR).

Antibodies—mAbs were prepared from female MRL/MpJ-Fas^{lpr} mice (The Jackson Laboratory, Bar Harbor, ME; 4–5 weeks) immunized with gp120-CRA **III** (23 mol of phosphonate diester/mol of gp120). The mice were injected intraperitoneally on days 0, 14, and 28 days with gp120-CRA **III** (11 μg) in Ribi adjuvant (monophosphoryl lipid A + trehalose dicorynomycolate emulsion; Sigma) followed by a fourth intravenous booster without adjuvant on day 55. Blood was obtained from the retroorbital plexus over the course of the immunization schedule. Three days after the final injection, hybridomas were prepared by fusion of splenocytes with myeloma cell line (NS-1; Ref. 3). After identification of wells secreting the desired Abs by ELISA, monoclonal cell lines were prepared by two rounds of cloning by limiting dilution. Monoclonal IgG was prepared from tissue culture supernatants containing mAbs (200 ml) by affinity chromatography on immobilized protein G (3). Control mAbs (anti-VIP clone c23.5 and anti-yellow fever virus antigen clone CRL 1689, ATCC) and serum IgG were purified similarly. The IgG preparations were electrophoretically homogeneous, determined by silver staining of overloaded IgG and immunoblotting with specific Abs to mouse IgG (3). Additional immunizations of female BALB/c mice (Jackson; 4–5 weeks) with gp120 or gp120-CRA were carried out similarly. mAb heavy and light chain isotypes were determined by ELISA as described (3).

ELISA—Maxisorp 96-well microtiter plates (Nunc) were coated with gp120 or gp120-CRA (40–100 ng/well) in 100 mM bicarbonate buffer, pH 8.6. Routine ELISAs were carried out as described (16). For assay of irreversible binding, the Abs were allowed to bind the plates, and the wells were treated for 30 min with 2% SDS in 10 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, 0.05% Tween 20, pH 7.4 (PBS-Tween) or PBS-Tween without SDS (control wells for measurement of total binding). The wells were then washed three times with PBS-Tween, and bound IgG was determined as usual using a peroxidase conjugate of goat anti-mouse IgG (Fc-specific; Sigma). Observed values of binding were corrected for nonspecific binding in wells containing nonimmune IgG or nonimmune mouse serum ($A_{490} \leq 0.03$). Percent residual binding in SDS-treated wells was computed as (A_{490}, SDS-treated wells) × 100/(A_{490}, PBS-Tween-treated wells).

Electrophoresis of Ab-CRA Complexes—Irreversible binding of biotinylated CRAs by purified IgG was determined by denaturing electrophoresis (6). Briefly, the reaction mixtures were incubated at 37 °C in 50 mM Tris-HCl, 0.1 M glycine, pH 8.0. SDS was added to 2%, and the mixtures were boiled (5 min) and then subjected to SDS-PAGE (4–20%, Bio-Rad, or 8–25% Phast gels, Amersham Biosciences). After electroblotting onto nitrocellulose membranes (0.22 μm, Bio-Rad), the membranes were blocked with 5% skim milk in PBS-Tween and processed for detection of IgG or biotin using peroxidase-conjugated goat anti-mouse IgG (Sigma) or peroxidase-conjugated streptavidin, respectively. Imaging and quantification were using x-ray film (Eastman Kodak Co.) with Unscan-it software (Silk scientific, Orem, UT) or a Fluoro-STM MultiImager (Bio-Rad). Biotinylated bovine serum albumin (11 mol of biotin/mol of bovine serum albumin, Sigma) was employed to construct a standard curve (0.06–1.5 pmol of biotin/lane).

Hydrolysis Assays—Biotinylated proteins were incubated with IgG in 50 mM Tris-HCl, 0.1 M glycine, 0.1 mM CHAPS, pH 8, at 37 °C. The reaction was terminated by addition of SDS to 2%, and the samples were boiled (5 min) and then analyzed by reducing SDS-gel electrophoresis (4–20%, Bio-Rad). Biotin containing protein bands in blots of the gel were identified and quantified as in the preceding section. In some blots, reaction products were identified by immunoblotting using peroxidase-conjugated goat anti-gp120 Abs (Fitzgerald, Concord, MA; catalog #60-H14) (16). N-terminal sequencing of protein bands from electrophoresis gels was done as described previously (17). Hydrolysis of peptide-MCA substrates (Peptide International, Louisville, KY or Bachem Biosciences, King of Prussia, PA) was determined in 96-well plates by fluorimetric detection of aminomethylcoumarin (Varian Cary Eclipse; λ_{ex} 360 nm, λ_{em} 470 nm) with authentic aminomethylcoumarin as standard (6). Cleavage of [Tyr^{10},^{125}I]VIP by mAb c23.5 was measured as the radioactivity rendered soluble in trichloroacetic acid (17). Kinetic parameters for cleavage of increasing concentrations of peptide-MCA substrates were determined from the Michaelis-Menten equation, $v = (V_{max}[S])/(K_m + [S])$. Because of the expense of studying gp120 cleavage at large concentrations of the protein, K_d (~ K_m) and k_{cat} for this reaction were obtained from the general quadratic equation (17) $[CS]^2 - [CS]([C_t] + [S_t] + K_d) + [C_t][S_t] = 0$, where $[C_t]$ and $[S_t]$ are the total concentrations of catalyst and substrate, and [CS] is the catalyst-substrate concentration. The method consists of calculation of [CS] at a series of assumed K_d values. The assumed K_d value yielding the best fit (by linear regression) between the observed reaction velocity and [CS] represents the experimentally determined K_d. k_{cat} is computed as the slope of the observed velocity *versus* [CS] plot.

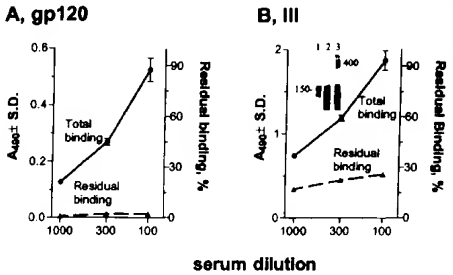

FIG. 2. **Irreversible III binding by polyclonal Abs.** A, immobilized gp120. *B*, immobilized **III**. Shown are ELISA values for binding of polyclonal Abs in serum of mice hyperimmunized with **III** (pooled sera, $n = 4$ mice). Binding of nonimmune mouse serum was negligible (A_{490} of 1:100 nonimmune serum in 0.001 (*A*) and −0.002 (*B*)). Residual and total binding represent A_{490} values in wells treated with and without SDS, respectively. *Inset*, anti-IgG stained blot of SDS-electrophoresis gels showing **III** (0.3 μM) treated for 48 h with nonimmune IgG (*lane 2*, 0.1 μM) and anti-**III** IgG (*lane 3*, 0.1 μM). Large Ab-containing adducts are evident at ~ 400 kDa in *lane 3*. *Lane 1* is a shorter exposure of *lane 2* showing a well defined 150-kDa band at the position of the smear evident in overexposed *lanes 2* and *3*.

RESULTS

gp120-CRA Design and Validation—Synthesis of hapten CRAs **I** and **II** (Fig. 1) and their covalent reactivity with naturally occurring proteolytic Abs has been described previously (6, 7). The electrophilic phosphonate mimics the peptide bond carbonyl group susceptible to nucleophilic attack, the positively charged amidino group adjacent to the phosphonate diester serves as a mimic of Lys/Arg P1 residues at which cleavage by germ line-encoded proteolytic Abs is observed (6), and the biotin group in **I** permits sensitive detection of Ab-phosphonate adducts. gp120-CRA **III** contains phosphonate diester groups in spatial proximity with antigenic epitopes presented by the protein. Multiple phosphonate diester groups were available per molecule of gp120, allowing presentation of the electrophilic hapten in conjunction with diverse antigenic epitopes.

Robust polyclonal Ab responses in MRL/lpr and BALB/c mice immunized with **III** were observed by routine ELISA. Abs raised to **III** were bound at somewhat greater levels by immobilized **III** than control gp120 devoid of phosphonate diester groups (Fig. 2). Conversely, Abs raised to control gp120 recognized immobilized **III**, but the binding was 3–4-fold lower than by immobilized gp120 (*e.g.* at serum dilution of 1:1000, A_{490} 0.44 ± 0.03 for immobilized **III** and 1.40 ± 0.03 for immobilized

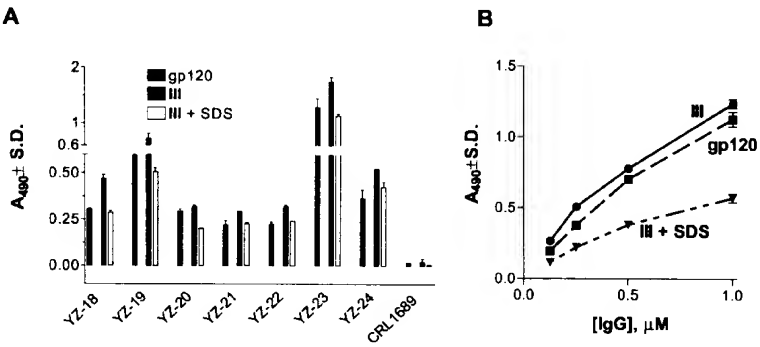

FIG. 3. **Irreversible III binding by monoclonal Abs.** ELISA showing SDS-resistant **III** binding by tissue culture supernatants containing mAbs (YZ series) (*A*) and monoclonal IgG purified from clone YZ18 (*B*) raised by immunization with gp120-CRA **III**. mAb CRL 1689 is an irrelevant monoclonal IgG with same isotype as mAbs YZ21 and YZ23. Immobilized antigens are gp120 and **III**. SDS-resistant **III** binding is indicated by *bars* and the curve labeled **III** + SDS.

gp120). **III** binding by nonimmune Abs was negligible, indicating that indiscriminate covalent binding at the hapten groups was not a problem. The observed differences in the antigenic reactivity of gp120 and **III** were held to be sufficiently small to proceed with further Ab studies. To facilitate high throughput screening, the feasibility of measuring irreversible **III** binding by Abs was studied by ELISA. After binding of polyclonal Abs anti-**III** Abs to the immobilized antigens, ELISA plates were treated with the denaturant SDS to remove reversibly bound Abs. SDS treatment allowed essentially complete removal of anti-**III** Abs bound by control gp120 devoid of hapten phosphonate groups. In comparison, 13–40% of the overall anti-**III** Ab binding activity consistently remained bound to immobilized **III** after SDS treatment in three repeat experiments. SDS-electrophoresis and immunoblotting with Abs to mouse IgG confirmed formation of irreversible Ab-**III** complexes in boiled reaction mixtures (Fig. 2*B*, *inset*, *lane 3*, estimated mass from extrapolated standard curve of molecular mass standards, ~400 kDa; large complexes can be formed by binding of multiple Abs to hapten groups in **III**).

Catalytic Activity—mAbs were prepared from MRL/lpr mice immunized with gp120-CRA **III**. This mouse strain develops lupus-like autoimmune disease attributable to the dysfunctional Fas-receptor gene. Spontaneous development of proteolytic Abs (18) and increased synthesis of esterase Abs in response to immunization with phosphonate monoester haptens (19, 20) have been reported in this mouse strain. Supernatants from 712 hybridoma wells (two splenocyte-myeloma cell fusions) were screened for SDS-resistant binding to **III**. IgG from seven wells was positive for this activity. After cloning of the cells by limiting dilution, monoclonal IgG from the supernatants of the seven cell lines was purified, and the binding assays were repeated (Fig. 3; clones YZ18, IgG2a,κ; YZ19, IgG2b,κ; YZ20, IgG2a,κ; YZ21, IgG2a,κ; YZ22, IgG2a,κ; YZ23, IgG2a,κ, and YZ24, IgG1,κ). Of total binding observed without SDS treatment of the ELISA plates, residual binding after the detergent treatment was 43–83% in 4 repeat assays. All seven mAbs were also bound by gp120 devoid of hapten CRA groups determined by routine ELISA without SDS treatment, indicating that they are not directed to neoepitopes generated by chemical modification procedures used for **III** preparation. An irrelevant mAb (clone CRL 1689) displayed no detectable binding of **III** or gp120.

Of seven mAbs with irreversible **III** binding activity, slow

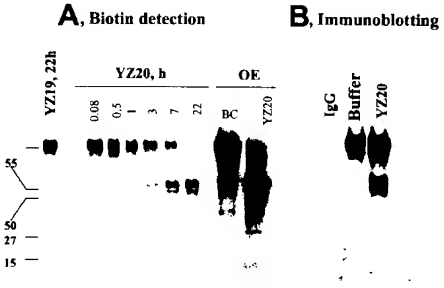

FIG. 4. **Cleavage of Bt-gp120 by mAb YZ20.** *A*, streptavidin peroxidase-stained blot of SDS-electrophoresis gels showing time-dependent Bt-gp120 cleavage by mAb YZ20 and lack of cleavage by mAb YZ19 (22 h incubation). IgG, 1 μM; Bt-gp120, 0.2 μM. *OE*, overexposed lanes showing Bt-gp120 incubated for 22 h in diluent and with YZ20 IgG (1 μM). Product bands at 27 and 15 kDa are visible in addition to the major 50–55 kDa bands. *B* anti-gp120-peroxidase stained blot of SDS-electrophoresis gel showing gp120 (1 μM) incubated with diluent or YZ20 IgG (1 μM, 24 h).

cleavage of Bt-gp120 by three mAbs was detected (YZ18, YZ20, YZ24), determined by the appearance of biotin-containing fragments of the protein in SDS-electrophoresis gels. The electrophoretic pattern of Bt-gp120 cleaved by mAbs YZ18 and YZ24 were similar to that shown for mAb YZ20 in Fig. 4. mAb YZ20 was further studied as it cleaved Bt-gp120 ~5-fold more rapidly than the other two mAbs. The consumption of gp120 was time-dependent (Fig. 4*A*). Major biotin-containing cleavage products with apparent mass 55 and 50 kDa were observed along with less intensely stained bands at 27 and 15 kDa. A band at 35 kDa was visible in overexposed gels, but this does not represent a product of mAb cleavage, as it was present at similar density in control incubations of Bt-gp120 in diluent. A control-irrelevant mAb (clone CRL 1689) did not cleave Bt-gp120. Immunoblotting using polyclonal anti-gp120 Abs confirmed that non-biotinylated gp120 is also susceptible to cleavage by the mAb (55-kDa cleavage product, Fig. 4*B*). Both detection methods allow quantification of gp120 cleavage by measuring depletion of intact gp120. Neither method provides guidance about the complete product profile or product concen-

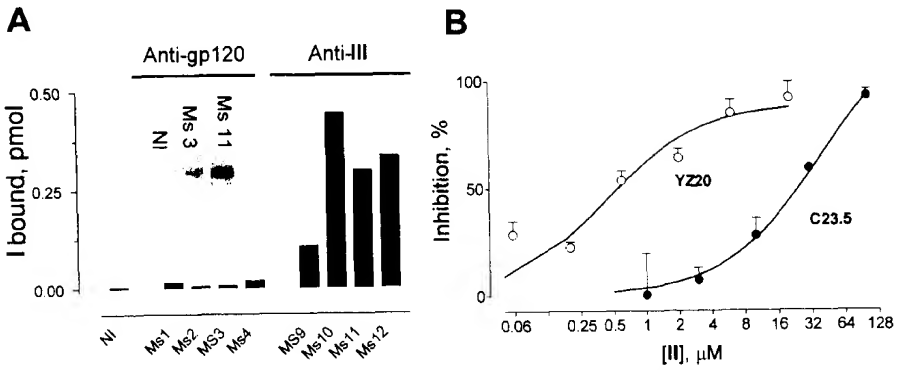

FIG. 6. **Enhanced hapten CRA I covalent binding by polyclonal IgG from mice immunized with III (*A*) and potent inhibition of mAb YZ20 cleavage of Bt-gp120 by hapten CRA II (*B*).** *A*, binding of hapten CRA **I** (10 μM), determined by incubation with IgG (0.4 μM) from BALB/c mice immunized with **III** (Ms9–12) or control gp120 (Ms1–4) for 60 min, SDS-electrophoresis, and quantification of the biotin-containing band at 150 kDa. *NI*, nonimmune IgG (pooled from 20 mice). *Inset*, representative SDS-electrophoresis lanes showing hapten CRA **I** binding by IgG from a mouse immunized with **III** (*Ms11*), a mouse immunized with gp120 (*Ms3*), and nonimmune IgG (*NI*). *B*, Bt-gp120 (0.1 μM) cleavage by mAb YZ20 (1 μM, 4 h) and [Tyr^{10}-^{125}I]VIP (~100 pM, 45,000 cpm) cleavage by mAb c23.5 (20 nM, 18 h) were measured in the presence of increasing **II** concentrations. In the absence of **II**, 15 and 40% of available Bt-gp120 and VIP, respectively, were cleaved.

CRA does not contain antigenic epitopes belonging to gp120, and noncovalent binding interactions are not anticipated to contribute to its irreversible binding by Abs. IgG samples from all four mice immunized with **III** displayed superior **I** binding compared with IgG from mice immunized with control gp120 (mean values, 0.31 and 0.01 pmol **I**; $p < 0.02$, Student's t test, unpaired observations) as well as pooled nonimmune IgG (Fig. 6). BALB/c mice were studied in this immunization. It may be concluded that synthesis of nucleophilic Abs in response to immunization with **III** is not restricted to autoimmune hosts (mAbs to gp120-CRA **III** were prepared from MRL/lpr mice).

DISCUSSION

The goal of this study was to strengthen the intrinsic serine protease-like reactivity of Abs and direct the reactivity to cleavage of gp120. Improved irreversible binding of hapten CRA by Abs after immunization with gp120-CRA **III** was evident, and the hapten CRA was a potent inhibitor of gp120 cleavage by a mAb. These observations suggest adaptive improvement of Ab nucleophilicity induced by the phosphonate diester groups. Specificity of the Abs for gp120 was obtained by traditional noncovalent mechanisms, *i.e.* recognition of gp120 epitopes located in the proximity of the phosphonate diester groups. No cleavage of unrelated proteins by the gp120-cleaving mAb was observed, and the K_m value of cleavage of a model peptide was 200-fold greater than of gp120 cleavage, indicating the absence of indiscriminate proteolysis.

Proteolysis entails Ab attack on the backbone of gp120, whereas the phosphonate electrophiles are located in Lys side chains of the immunogen. Because mAbs raised to gp120-CRA displayed proteolytic activity, the nucleophile developed to recognize the side chain electrophiles must enjoy sufficient conformational freedom to attack the polypeptide backbone. Movements of individual amino acids in Ab combining sites after binding to antigen have been reported (24, 25). Epitope mapping and mutagenesis studies of certain proteolytic Abs indicate that the catalytic residues do not participate in stabilization of the Ab-antigen ground state complex (26, 27), suggesting that the mobility of the nucleophile may not be restricted by initial noncovalent Ab-antigen interactions. Naturally occurring mAbs to VIP (17) and gp41 (4) cleave multiple peptide bonds in these antigens, which may be explained by hypothesizing formation of alternate transition states in which the nucleophile is free to initiate nucleophilic attack on spatially neighboring peptide bonds (for review, see Ref. 28). Understanding the extent of conformational freedom of Ab nucleophiles is important, because there is no viable alternative to locating the peptide bond mimetic in the side chains when large proteins must be used to induce the synthesis of catalytic Abs. In addition to direct structural analysis of nucleophile movements in available catalytic Abs, the length and flexibility of the linker utilized to attach the phosphonate groups at Lys side chains can be varied in future studies to assess the flexibility of the catalytic site. In the case of synthetic peptide immunogens, the phosphonate groups can be incorporated within the peptide backbone to better mimic the intended scissile bond (7). However, synthetic peptides often fail to assume conformations similar to their cognate determinants in full-length proteins, in which case anti-peptide Abs do not recognize the parent proteins.

The fully competent catalytic machinery found in modern non-Ab serine proteases has presumably evolved in response to selection pressures that optimized each of the rate-limiting steps in the catalytic cycle. In comparison, a CRA immunogen can at best select for Abs with the greatest covalent attack capability. No selection for hydrolysis of the acyl-Ab complex or the subsequent product release steps is anticipated, which may account for observations of limited Ab turnover. Two previous attempts to raise esterase Abs indicated the formation of irreversible substrate binding by Abs (29, 30), suggesting the need to optimize events occurring after nucleophilic attack by the Abs. Furthermore, structural refinements of the immunogen could be implemented to help guide the Ab-antigen complex toward the catalytic pathway, *e.g.* inclusion of a component that binds a water molecule and facilitates hydrolysis of the acyl-protein complexes. Notwithstanding this weakness, the results reported here represent a significant advance toward developing antigen specific proteolytic Abs. Previously, several Abs with haptenic ester-hydrolyzing activity have been raised

based on the premise that catalytic sites capable of noncovalent stabilization of the oxyanionic transition states can be formed *de novo* over the course of adaptive sequence diversification of Ab variable domains (8, 9). This approach has not been successful for development of proteolytic Abs. Pollack *et al.* (31) describe the failure of a phosphonate monoester analog of Phe-Leu-Ala to induce proteolytic Ab synthesis. No attempt was made in this study to recruit the intrinsic properties of natural Abs for the purpose of protease synthesis, *i.e.* their nucleophilicity and selective recognition of basic residues adjacent to the cleavage site. Recently, phosphonate monoesters were discovered to form covalent bonds with nucleophiles in serine proteases, but their reactivity is weaker than the diester used in the present study, and no detectable reaction occurs unless an adjacent positive charge is present (6, 12).

Evidence for increased potency because of the catalytic function has recently been published in regard to Ab antagonism of the biological effects of VIP, a 28-amino acid neuropeptide (32, 33). Concerning gp120, a major hurdle has been to induce the synthesis of Abs that recognize the determinants involved in viral entry, *i.e.* the binding sites for host CD4 and chemokine receptors. Most Abs raised to monomer gp120 are directed to its variable region epitopes, and the Abs do not neutralize diverse HIV-1 strains found in different geographical locations (34). Reversibly binding Abs must bind at or near the receptor binding sites of gp120 to sterically hinder HIV entry into host cells. Proteolytic Abs offer the potential advantage of gp120 inactivation even if cleavage occurs at a site that does not itself participate in binding to host cells. Discussion of the immunotherapeutic potential of mAbs to gp120-CRA **III** is beyond the scope of the present study, but initial HIV-1 neutralization studies suggest that certain mAbs raised to gp120-CRA **III** neutralize the HIV-1 primary isolate ZA009 (peripheral blood mononuclear cell cultures; infection was measured by determining p24 antigen concentrations).[2] A potential pitfall is that proteolytic Abs to monomer gp120-CRA may not recognize trimeric gp120 on the surface of HIV-1, as observed for reversibly binding Abs to the protein (35). The CRA immunogen techniques described in the present study are readily applicable to recently developed recombinant mimetics of trimeric gp120 (36) as well as whole HIV-1 particles.

Acknowledgment—We thank the Monoclonal Antibody Core Facility of the University of Nebraska for preparing hybridomas.

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